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⑯ **Fusion proteins containing N-terminal fragments of human serum albumin.**

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**EP-A- 0 308 381
EP-A- 0 322 094**

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Description

The present invention relates to fusion polypeptides where two individual polypeptides or parts thereof are fused to form a single amino acid chain. Such fusion may arise from the expression of a single continuous coding sequence formed by recombinant DNA techniques.

Fusion polypeptides are known, for example those where a polypeptide which is the ultimately desired product of the process is expressed with an N-terminal "leader sequence" which encourages or allows secretion of the polypeptide from the cell. An example is disclosed in EP-A-116 201 (Chiron).

Human serum albumin (HSA) is a known protein found in the blood. EP-A-147 198 (Delta Biotechnology) discloses its expression in a transformed host, in this case yeast. Our earlier application EP-A-322 094 discloses N-terminal fragments of HSA, namely those consisting of residues 1-n where n is 369 to 419, which have therapeutic utility. The application also mentions the possibility of fusing the C-terminal residue of such molecules to other, unnamed, polypeptides.

One aspect of the present invention provides a fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor, or a variant thereof, (d) transforming growth factor, or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.

The N-terminal portion of HSA is preferably the said 1-n portion, the 1-177 portion (up to and including the cysteine), the 1-200 portion (up to but excluding the cysteine) or a portion intermediate 1-177 and 1-200.

The term "human serum albumin" (HSA) is intended to include (but not necessarily to be restricted to) known or yet-to-be-discovered polymorphic forms of HSA. For example, albumin Naskapi has Lys-372 in place of Glu-372 and pro-albumin Christchurch has an altered pro-sequence. The term "variants" is intended to include (but not necessarily to be restricted to) minor artificial variations in sequence (such as molecules lacking one or a few residues, having conservative substitutions or minor insertions of residues, or having minor variations of amino acid structure). Thus polypeptides which have 80%, preferably 85%, 90%, 95% or 99%, homology with HSA are deemed to be "variants". It is also preferred for such variants to be physiologically equivalent to HSA; that is to say, variants preferably share at least one pharmacological utility with HSA. Furthermore, any putative variant which is to be used pharmacologically should be non-immunogenic in the animal (especially human) being treated.

Conservative substitutions are those where one or more amino acids are substituted for others having similar properties such that one skilled in the art of polypeptide chemistry would expect at least the secondary structure, and preferably the tertiary structure, of the polypeptide to be substantially unchanged. For example, typical such substitutions include asparagine for glutamine, serine for asparagine and arginine for lysine. Variants may alternatively, or as well, lack up to ten (preferably only one or two) intermediate amino acid residues (ie not at the termini of the said N-terminal portion of HSA) in comparison with the corresponding portion of natural HSA; preferably any such omissions occur in the 100 to 369 portion of the molecule (relative to mature HSA itself) (if present). Similarly, up to ten, but preferably only one or two, amino acids may be added, again in the 100 to 369 portion for preference (if present). The term "physiologically functional equivalents" also encompasses larger molecules comprising the said sequence plus a further sequence at the N-terminal (for example, pro-HSA, pre-pro-HSA and met-HSA).

Clearly, the said "another polypeptide" in the fusion compounds of the invention cannot be the remaining portion of HSA, since otherwise the whole polypeptide would be HSA, which would not then be a "fusion polypeptide".

Even when the HSA-like portion is not the said 1-n portion of HSA, it is preferred for the non-HSA portion to be one of the said (a) to (h) entities.

The 1 to 368 portion of CD4 represents the first four disulphide-linked immunoglobulin-like domains of the human T lymphocyte CD4 protein, the gene for and amino acid sequence of which are disclosed in D. Smith *et al* (1987) *Science* **328**, 1704-1707. It is used to combat HIV infections.

The sequence of human platelet-derived growth factor (PDGF) is described in Collins *et al* (1985) *Nature* **316**, 748-750. Similarly, the sequence of transforming growth factors β (TGF- β) is described in Deryck *et al* (1985) *Nature* **316**, 701-705. These growth factors are useful for wound-healing.

A cDNA sequence for the 1-261 portion of Fn was disclosed in EP-A-207 751 (obtained from plasmid pFH6 with endonuclease PvuII). This portion binds fibrin and can be used to direct fused compounds to blood clots.

A cDNA sequence for the 278-578 portion of Fn, which contains a collagen-binding domain, was disclosed by R.J. Owens and F.E. Baralle in 1986 E.M.B.O.J. 5, 2825-2830. This portion will bind to platelets.

The 1-272 portion of von Willebrand's Factor binds and stabilises factor VIII. The sequence is given in Bontham *et al.* Nuc. Acids Res. 14, 7125-7127.

5 Variants of alpha-1-antitrypsin include those disclosed by Rosenburg *et al* (1984) Nature 312, 77-80. In particular, the present invention includes the Pittsburgh variant (Met³⁵⁸ is mutated to Arg) and the variant where Pro³⁵⁷ and Met³⁵⁸ are mutated to alanine and arginine respectively. These compounds are useful in the treatment of septic shock and lung disorders.

10 Variants of the non-HSA portion of the polypeptides of the invention include variations as discussed above in relation to the HSA portion, including those with conservative amino acid substitutions, and also homologues from other species.

15 The fusion polypeptides of the invention may have N-terminal amino acids which extend beyond the portion corresponding to the N-terminal portion of HSA. For example, if the HSA-like portion corresponds to an N-terminal portion of mature HSA, then pre-, pro-, or pre-pro sequences may be added thereto, for example the yeast alpha-factor leader sequence. The fused leader portions of WO 90/01063 may be used. The polypeptide which is fused to the HSA portion may be a naturally-occurring polypeptide, a fragment thereof or a novel polypeptide, including a fusion polypeptide. For example, in Example 3 below, a fragment of fibronectin is fused to the HSA portion via a 4 amino acid linker.

20 It has been found that the amino terminal portion of the HSA molecule is so structured as to favour particularly efficient translocation and export of the fusion compounds of the invention in eukaryotic cells.

25 A second aspect of the invention provides a transformed host having a nucleotide sequence so arranged as to express a fusion polypeptide as described above. By "so arranged", we mean, for example, that the nucleotide sequence is in correct reading frame with an appropriate RNA polymerase binding site and translation start sequence and is under the control of a suitable promoter. The promoter may be homologous with or heterologous to the host. Downstream (3') regulatory sequences may be included if desired, as is known. The host is preferably yeast (for example Saccharomyces spp., e.g. S. cerevisiae; Kluyveromyces spp., e.g. K. lactis; Pichia spp.; or Schizosaccharomyces spp., e.g. S. pombe) but may be any other suitable host such as E. coli, B. subtilis, Aspergillus spp., mammalian cells, plant cells or insect cells.

30 A third aspect of the invention provides a process for preparing a fusion polypeptide according to the first aspect of the invention by cultivation of a transformed host according to the second aspect of the invention, followed by separation of the fusion polypeptide in a useful form.

35 A fourth aspect of the invention provides therapeutic methods of treatment of the human or other animal body comprising administration of such a fusion polypeptide.

40 In the methods of the invention we are particularly concerned to improve the efficiency of secretion of useful therapeutic human proteins from yeast and have conceived the idea of fusing to amino-terminal portions of HSA those proteins which may ordinarily be only inefficiently secreted. One such protein is a potentially valuable wound-healing polypeptide representing amino acids 585 to 1578 of human fibronectin (referred to herein as Fn 585-1578). As we have described in a separate application (filed simultaneously herewith) this molecule contains cell spreading, chemotactic and chemokinetic activities useful in healing wounds. The fusion polypeptides of the present invention wherein the C-terminal portion is Fn 585-1578 can be used for wound healing applications as biosynthesised, especially where the hybrid human protein will be topically applied. However, the portion representing amino acids 585 to 1578 of human fibronectin can if desired be recovered from the fusion protein by preceding the first amino acid of the fibronectin portion by amino acids comprising a factor X cleavage site. After isolation of the fusion protein from culture supernatant, the desired molecule is released by factor X cleavage and purified by suitable chromatography (e.g. ion-exchange chromatography). Other sites providing for enzymatic or chemical cleavage can be provided, either by appropriate juxtaposition of the N-terminal and C-terminal portions or by the insertion therebetween of an appropriate linker.

45 At least some of the fusion polypeptides of the invention, especially those including the said CD4 and vWF fragments, PDGF and α ,AT, also have an increased half-life in the blood and therefore have advantages and therapeutic utilities themselves, namely the therapeutic utility of the non-HSA portion of the molecule. In the case of α ,AT and others, the compound will normally be administered as a one-off dose or only a few doses over a short period, rather than over a long period, and therefore the compounds are less likely to cause an immune response.

55 EXAMPLES : SUMMARY

Standard recombinant DNA procedures were as described by Maniatis *et al* (1982 and recent 2nd edition) unless otherwise stated. Construction and analysis of phage M13 recombinant clones was as described by

Messing (1983) and Sanger et al (1977).

5 DNA sequences encoding portions of human serum albumin used in the construction of the following molecules are derived from the plasmids mHOB12 and pDBD2 (EP-A-322 094, Delta Biotechnology Ltd, relevant portions of which are reproduced below) or by synthesis of oligonucleotides equivalent to parts of this sequence. DNA sequences encoding portions of human fibronectin are derived from the plasmid pFHDEL1, or by synthesis of oligonucleotides equivalent to parts of this sequence. Plasmid pFHDEL1, which contains the complete human cDNA encoding plasma fibronectin, was obtained by ligation of DNA derived from plasmids pFH6, 16, 54, 154 and 1 (EP-A-207 751; Delta Biotechnology Ltd).

10 This DNA represents an mRNA variant which does not contain the 'ED' sequence and had an 89-amino acid variant of the III-CS region (R.J. Owens, A.R. Kornblihtt and F.E. Baralle (1986) Oxford Surveys on Eukaryotic Genes 3 141-160). The map of this vector is disclosed in Fig. 11 and the protein sequence of the mature polypeptide produced by expression of this cDNA is shown in Fig. 5.

15 Oligonucleotides were synthesised on an Applied Biosystems 380B oligonucleotide synthesiser according to the manufacturer's recommendations (Applied Biosystems, Warrington, Cheshire, UK).

20 An expression vector was constructed in which DNA encoding the HSA secretion signal and mature HSA up to and including the 387th amino acid, leucine, fused in frame to DNA encoding a segment of human fibronectin representing amino acids 585 to 1578 inclusive, was placed downstream of the hybrid promoter of EP-A-258 067 (Delta Biotechnology), which is a highly efficient galactose-inducible promoter functional in Saccharomyces cerevisiae. The codon for the 1578th amino acid of human fibronectin was directly followed by a stop codon (TAA) and then the S. cerevisiae phosphoglycerate kinase (PGK) gene transcription terminator. This vector was then introduced into S. cerevisiae by transformation, wherein it directed the expression and secretion from the cells of a hybrid molecule representing the N-terminal 387 amino acids of HSA C-terminally fused to amino acids 585 to 1578 of human fibronectin.

25 In a second example a similar vector is constructed so as to enable secretion by S. cerevisiae of a hybrid molecule representing the N-terminal 195 amino acids of HSA C-terminally fused to amino acids 585 to 1578 of human fibronectin.

30 Aspects of the present invention will now be described by way of example and with reference to the accompanying drawings, in which:

35 Figure 1 (on two sheets) depicts the amino acid sequence currently thought to be the most representative of natural HSA, with (boxed) the alternative C-termini of HSA(1-n);

Figure 2 (on two sheets) depicts the DNA sequence coding for mature HSA, wherein the sequence included in Linker 3 is underlined;

Figure 3 illustrates, diagrammatically, the construction of mHOB16;

Figure 4 illustrates, diagrammatically, the construction of pHOB31;

40 Figure 5 (on 6 sheets) illustrates the mature protein sequence encoded by the Fn plasmid pFHDEL1;

Figure 6 illustrates Linker 5, showing the eight constituent oligonucleotides;

Figure 7 shows schematically the construction of plasmid pDBDF2;

Figure 8 shows schematically the construction of plasmid pDBDF5;

Figure 9 shows schematically the construction of plasmid pDBDF9;

45 Figure 10 shows schematically the construction of plasmid DBDF12, using plasmid pFHDEL1; and

Figure 11 shows a map of plasmid pFHDEL1.

EXAMPLE 1 : HSA 1-387 FUSED TO Fn 585-1578

45 The following is an account of a preparation of plasmids comprising sequences encoding a portion of HSA, as is disclosed in EP-A-322 094.

50 The human serum albumin coding sequence used in the construction of the following molecules is derived from the plasmid M13mp19.7 (EP-A-201 239, Delta Biotechnology Ltd.) or by synthesis of oligonucleotides equivalent to parts of this sequence. Oligonucleotides were synthesised using phosphoramidite chemistry on an Applied Biosystems 380B oligonucleotide synthesizer according to the manufacturer's recommendations (AB Inc., Warrington, Cheshire, England).

An oligonucleotide was synthesised (Linker A) which represented a part of the known HSA coding sequence (Figure 2) from the PstI site (1235-1240, Figure 2) to the codon for valine 381 wherein that codon was changed from GTG to GTC:

Linker 1

		D	P	H	E	C	Y	
5	5'	GAT	CCT	CAT	GAA	TGC	TAT	
	3'	ACGT	CTA	GGA	GTA	CTT	ACG	
1247								
10								
	A	K	V	F	D	E	F	K
15	GCC	AAA	GTG	TTC	GAT	GAA	TTT	AAA
	CGG	TTT	CAC	AAG	CTA	CTT	AAA	TTT
1267								
20								
	P	L	V					
	CTT	GTC	3'					
25	GGA	CAG	5'					

Linker 1 was ligated into the vector M13mp19 (Norrander *et al*, 1983) which had been digested with PstI and HincII and the ligation mixture was used to transfect E.coli strain XL1-Blue (Stratagene Cloning Systems, San Diego, CA). Recombinant clones were identified by their failure to evolve a blue colour on medium containing the chromogenic indicator X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in the present of IPTG (isopropylthio-β-galactoside). DNA sequence analysis of template DNA prepared from bacteriophage particles of recombinant clones identified a molecule with the required DNA sequence, designated mHOB12 (Figure 3). M13mp19.7 consists of the coding region of mature HSA in M13mp19 (Norrander *et al*, 1983) such that the codon for the first amino acid of HSA, GAT, overlaps a unique Xhol site thus:

		Asp	Ala	
35	5'	C T C G A G A T G C A		3'
40	3'	G A G C T C T A C G T		5'
<u>Xhol</u>				

45 (EP-A-210 239). M13mp19.7 was digested with Xhol and made flush-ended by S1-nuclease treatment and was then ligated with the following oligonucleotide (Linker 2):

Linker 2

50	5'	T C T T T T A T C C A A G C T T G G A T A A A A G A	3'
	3'	A G A A A A T A G G T T C G A A C C T A T T T C T	5'
<u>HindIII</u>			

The ligation mix was then used to transfect E.coli XL1-Blue and template DNA was prepared from several

plaques and then analysed by DNA sequencing to identify a clone, pDBD1 (Figure 4), with the correct sequence.

5 A 1.1 kb HindIII to PstI fragment representing the 5' end of the HSA coding region and one half of the inserted oligonucleotide linker was isolated from pDBD1 by agarose gel electrophoresis. This fragment was then ligated with double stranded mHOB12 previously digested with HindIII and PstI and the ligation mix was then used to transfect E.coli XL1-Blue. Single stranded template DNA was prepared from mature bacteriophage particles of several plaques. The DNA was made double stranded *in vitro* by extension from annealed sequencing primer with the Klenow fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates. Restriction enzyme analysis of this DNA permitted the identification of a clone with the correct configuration, 10 mHOB15 (Figure 4).

15 The following oligonucleotide (Linker 3) represents from the codon for the 382nd amino acid of mature HSA (glutamate, GAA) to the codon for lysine 389 which is followed by a stop codon (TAA) and a HindIII site and then a BamHI cohesive end:

15 Linker 3

	E	E	P	Q	N	L	I	K	J			
20	5'	GAA	GAG	CCT	CAG	AAT	TTA	ATC	AAA	TAA	GCTTG	3'
	3'	CTT	CTC	GGA	GTC	TTA	AAT	TAG	TTT	ATT	CGAACCTAG	5'

25 This was ligated into double stranded mHOB15, previously digested with HincII and BamHI. After ligation, the DNA was digested with HincII to destroy all non-recombinant molecules and then used to transfect E.coli XL1-Blue. Single stranded DNA was prepared from bacteriophage particles of a number of clones and subjected to DNA sequence analysis. One clone having the correct DNA sequence was designated mHOB16 (Figure 4).

30 A molecule in which the mature HSA coding region was fused to the HSA secretion signal was created by insertion of Linker 4 into BamHI and XbaII digested M13mp19.7 to form pDBD2 (Figure 4).

Linker 4

		M	K	W	V	S	F		
35	5'	GATCC	ATG	AAG	TGG	GTA	AGC	TTT	
	40	G	TAC	TTC	ACC	CAT	TCG	AAA	
	45	I	S	L	L	F	L	F	S
		ATT	TCC	CTT	CTT	TTT	CTC	TTT	AGC
	50	TAA	AGG	GAA	GAA	AAA	GAG	AAA	TCG

	S	A	Y	S	R	G	V	F
5	TCG	GCT	TAT	TCC	AGG	GGT	GTG	TTT
	AGC	CGA	ATA	AGG	TCC	CCA	CAC	AAA

10 R R
CG 3'
GCAGCT 5'

15 In this linker the codon for the fourth amino acid after the initial methionine, ACC for threonine in the HSA pre-pro leader sequence (Lawn *et al.*, 1981), has been changed to AGC for serine to create a HindIII site.

20 A sequence of synthetic DNA representing a part of the known HSA coding sequence (Lawn *et al.*, 1981) (amino acids 382 to 387, Fig. 2), fused to part of the known fibronectin coding sequence (Kornblhtt *et al.*, 1985) (amino acids 585 to 640, Fig. 2), was prepared by synthesising six oligonucleotides (Linker 5, Fig. 6). The oligonucleotides 2, 3, 4, 6, 7 and 8 were phosphorylated using T4 polynucleotide kinase and then the oligonucleotides were annealed under standard conditions in pairs, i.e. 1+8, 2+7, 3+6 and 4+5. The annealed oligonucleotides were then mixed together and ligated with mHOB12 which had previously been digested with the restriction enzymes HincII and EcoRI. The ligation mixture was then used to transfect E.coli XL1-Blue (Stratagene Cloning Systems, San Diego, CA). Single stranded template DNA was then prepared from mature bacteriophage particles derived from several independent plaques and then was analysed by DNA sequencing. A clone in which a linker of the expected sequence had been correctly inserted into the vector was designated pDBDF1 (Fig. 7). This plasmid was then digested with PstI and EcoRI and the approx. 0.24kb fragment was purified and then ligated with the 1.29kb BamHI-PstI fragment of pDBD2 (Fig. 7) and BamHI + EcoRI digested pUC19 (Yanisch-Perron, *et al.*, 1985) to form pDBDF2 (Fig. 7).

25 A plasmid containing a DNA sequence encoding full length human fibronectin, pFHDEL1, was digested with EcoRI and XbaI and a 0.77kb EcoRI-XbaI fragment (Fig. 8) was isolated and then ligated with EcoRI and XbaI digested M13 mp18 (Norrandar *et al.*, 1983) to form pDBDF3 (Fig. 8).

30 The following oligonucleotide linker (Linker 6) was synthesised, representing from the PstI site at 4784-35 4791 of the fibronectin sequence of EP-A-207 751 to the codon for tyrosine 1578 (Fig. 5) which is followed by a stop codon (TAA), a HindIII site and then a BamHI cohesive end:

Linker 6

40 G P D Q T E M T I E G L
GGT CCA GAT CAA ACA GAA ATG ACT ATT GAA GGC TTG
45 A CGT CCA GGT CTA GTT TGT CTT TAC TGA TAA CTT CCG AAC

50 Q P T V E Y Stop
CAG CCC ACA GTG GAG TAT TAA GCTTG
GTC GGG TGT CAC CTC ATA ATT CGAACCTAG

55 This linker was then ligated with PstI and HindIII digested pDBDF3 to form pDBDF4 (Fig. 8). The following DNA fragments were then ligated together with BglII digested pKV50 (EP-A-258 067) as shown in Fig. 8: 0.68kb EcoRI-BamHI fragment of pDBDF4, 1.5kb BamHI-StuI fragment of pDBD2 and the 2.2kb StuI-EcoRI fragment of pFHDEL1. The resultant plasmid pDBDF5 (Fig. 8) includes the promoter of EP-A-258 067 to direct the ex-

pression of the HSA secretion signal fused to DNA encoding amino acids 1-387 of mature HSA, in turn fused directly and in frame with DNA encoding amino acids 585-1578 of human fibronectin, after which translation would terminate at the stop codon TAA. This is then followed by the S.cerevisiae PGK gene transcription terminator. The plasmid also contains sequences which permit selection and maintenance in Escherichia coli and S.cerevisiae (EP-A-258 067).

This plasmid was introduced into S.cerevisiae S150-2B (leu2-3 leu2-112 ura3-52 trp1-289 his3-1) by standard procedures (Beggs, 1978). Transformants were subsequently analysed and found to produce the HSA-fibronectin fusion protein.

10 EXAMPLE 2 : HSA 1-195 FUSED TO Fn 585-1578

In this second example the first domain of human serum albumin (amino acids 1-195) is fused to amino acids 585-1578 of human fibronectin.

15 The plasmid pDBD2 was digested with BamHI and BglII and the 0.79kb fragment was purified and then ligated with BamHI-digested M13mp19 to form pDBDF6 (Fig. 6). The following oligonucleotide:

5'-C C A A A G C T C G A G G A A C T T C G-3'

20 was used as a mutagenic primer to create a Xhol site in pDBDF6 by in vitro mutagenesis using a kit supplied by Amersham International PLC. This site was created by changing base number 696 of HSA from a T to a G (Fig. 2). The plasmid thus formed was designated pDBDF7 (Fig. 9). The following linker was then synthesised to represent from this newly created Xhol site to the codon for lysine 195 of HSA (AAA) and then from the codon for isoleucine 585 of fibronectin to the ends of oligonucleotides 1 and 8 shown in Fig. 6.

25 Linker 7

D	E	L	R	D	E	G	K	A	S	S	A	K
TC GAT GAA CTT CGG GAT GAA GGG AAG GCT TCG TCT GCC AAA												
A CTT GAA GCC CTA CTT CCC TTC CGA AGC AGA CGG TTT												
I	T	E	T	P	S	Q	P	N	S	H		
ATC ACT GAG ACT CCG AGT CAG C												
TAG TGA CTC TGA GGC TCA GTC GGG TTG AGG GTG G												

35 This linker was ligated with the annealed oligonucleotides shown in Fig. 3, i.e. 2+7, 3+6 and 4+5 together with Xhol and EcoRI digested pDBDF7 to form pDBDF8 (Fig. 9). Note that in order to recreate the original HSA DNA sequence, and hence amino acid sequence, insertion of linker 7 and the other oligonucleotides into pDBDF7 does not recreate the Xhol site.

40 The 0.83kb BamHI-StuI fragment of pDBDF8 was purified and then was ligated with the 0.68kb EcoRI-BamHI fragment of pDBDF2 and the 2.22kb StuI-EcoRI fragment of pFHDEL1 into BglII-digested pKV50 to form pDBDF9 (Fig. 9). This plasmid is similar to pDBDF5 except that it specifies only residues 1-195 of HSA rather than 1-387 as in pDBDF5.

45 When introduced into S.cerevisiae S150-2B as above, the plasmid directed the expression and secretion of a hybrid molecule composed of residues 1-195 of HSA fused to residues 585-1578 of fibronectin.

50 EXAMPLE 3 : HSA 1-387 FUSED TO Fn 585-1578, AS CLEAVABLE MOLECULE

55 In order to facilitate production of large amounts of residues 585-1578 of fibronectin, a construct was made in which DNA encoding residues 1-387 of HSA was separated from DNA encoding residues 585-1578 of fibronectin by the sequence

I E G R
ATT GAA GGT AGA
5 TAA CTT CCA TCT

10 which specifies the cleavage recognition site for the blood clotting Factor X. Consequently the purified secreted product can be treated with Factor X and then the fibronectin part of the molecule can be separated from the HSA part.

To do this two oligonucleotides were synthesised and then annealed to form Linker 8.

Linker 8

15	E	E	P	Q	N	L	I	E	G
	GAA	GAG	CCT	CAG	AAT	TTA	ATT	GAA	GGT
20	CTT	CTC	GGA	GTC	TTA	AAT	TAA	CTT	CCA
	R	I	T	E	T	P	S	Q	P
25	AGA	ATC	ACT	GAG	ACT	CCG	AGT	CAG	C
	TCT	TAG	TGA	CTC	TGA	GGC	TCA	GTC	GGG
30	N	S	H						
35	TTG	AGG	GTG	G					

40 This linker was then ligated with the annealed oligonucleotides shown in Fig. 6, i.e. 2+7, 3+6 and 4+5 into HincII and EcoRI digested mHOB12, to form pDBDF10 (Fig. 7). The plasmid was then digested with PstI and EcoRI and the roughly 0.24kb fragment was purified and then ligated with the 1.29kb BamHI-PstI fragment of pDBD2 and BamHI and EcoRI digested pUC19 to form pDBDF11 (Fig. 10).

45 The 1.5kb BamHI-StuI fragment of pDBDF11 was then ligated with the 0.68kb EcoRI-BamH1 fragment of pDBDF4 and the 2.22kb StuI-EcoRI fragment of pFHDEL1 into BglII-digested pKV50 to form pDBDF12 (Fig. 10). This plasmid was then introduced into S.cerevisiae S150-2B. The purified secreted fusion protein was treated with Factor X to liberate the fibronectin fragment representing residues 585-1578 of the native molecule.

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Claims**Claims for the following Contracting States : AT, BE, CH, LI, DE, DK, FR, IT, LU, NL, SE**

5 1. A fusion polypeptide comprising, as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor or a variant thereof, (d) transforming growth factor β or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.

10 2. A fusion polypeptide according to Claim 1 additionally comprising at least one N-terminal amino acid extending beyond the portion corresponding to the N-terminal portion of HSA.

15 3. A fusion polypeptide according to Claim 1 or 2 wherein there is a cleavable region at the junction of the said N-terminal or C-terminal portions.

20 4. A fusion polypeptide according to any one of the preceding claims wherein the said C-terminal portion is the 585 to 1578 portion of human plasma fibronectin or a variant thereof.

25 5. A transformed or transfected host having a nucleotide sequence so arranged as to express a fusion polypeptide according to any one of the preceding claims.

30 6. A process for preparing a fusion polypeptide by cultivation of a host according to Claim 5, followed by separation of the fusion polypeptide in a useful form.

35 7. A fusion polypeptide according to any one of Claims 1 to 4 for use in therapy.

Claims for the following Contracting States : ES, GR

1. A process for preparing a fusion polypeptide by (i) cultivation of a transformed or transfected host having a nucleotide sequence so arranged as to express a fusion polypeptide, followed by (ii) separation of the fusion polypeptide in a useful form, characterised in that the fusion polypeptide comprises as at least part of the N-terminal portion thereof, an N-terminal portion of HSA or a variant thereof and, as at least part of the C-terminal portion thereof, another polypeptide except that, when the said N-terminal portion of HSA is the 1-n portion where n is 369 to 419 or a variant thereof then the said polypeptide is (a) the 585 to 1578 portion of human fibronectin or a variant thereof, (b) the 1 to 368 portion of CD4 or a variant thereof, (c) platelet derived growth factor or a variant thereof, (d) transforming growth factor β or a variant thereof, (e) the 1-261 portion of mature human plasma fibronectin or a variant thereof, (f) the 278-578 portion of mature human plasma fibronectin or a variant thereof, (g) the 1-272 portion of mature human von Willebrand's Factor or a variant thereof, or (h) alpha-1-antitrypsin or a variant thereof.

45 2. A process according to Claim 1, wherein the fusion polypeptide additionally comprising at least one N-terminal amino acid extending beyond the portion corresponding to the N-terminal portion of HSA.

50 3. A process according to Claim 1 or 2 wherein, in the fusion polypeptide, there is a cleavable region at the junction of the said N-terminal or C-terminal portions.

55 4. A process according to any one of the preceding claims wherein the said C-terminal portion is the 585 to 1578 portion of human plasma fibronectin or a variant thereof.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, DK, FR, IT, LU, NL, SE

- 5 1. Fusionspolypeptid, umfassend als mindestens einen Teil seines N-terminalen Teils einen N-terminalen Teil von HSA oder eine Variante davon und als mindestens einen Teil seines C-terminalen Teils ein weiteres Polypeptid mit der Ausnahme, daß wenn es sich bei dem N-terminalen Teil von HSA um den Teil 1-n mit n = 369 bis 419 oder eine Variante davon handelt, das Polypeptid aus
 - (a) dem Teil 585 bis 1578 von Humanfibronectin oder einer Variante davon,
 - (b) dem Teil 1 bis 368 von CD4 oder einer Variante davon,
 - (c) dem "Platelet Derived Growth Factor" (PDGF) oder einer Variante davon,
 - (d) dem "Transforming Growth Factor β " (TGF β) oder einer Variante davon,
 - (e) dem Teil 1-261 von reifem Humanplasmafibronectin oder einer Variante davon,
 - (f) dem Teil 278-578 von reifem Humanplasmafibronectin oder einer Variante davon,
 - (g) dem Teil 1-272 von reifem Human-von Willebrand's-Faktor oder einer Variante davon oder
 - (h) Alpha-1-Antitrypsin oder einer Variante davon, besteht.
- 10 2. Fusionspolypeptid nach Anspruch 1, zusätzlich umfassend mindestens eine N-terminale Aminosäure, die länger als der dem N-terminalen Teil von HSA entsprechende Teil ist.
- 15 3. Fusionspolypeptid nach Anspruch 1 oder 2, bei dem sich an der Verbindung der N-terminalen oder C-terminalen Teile eine spaltbare Region befindet.
- 20 4. Fusionspolypeptid nach einem der vorhergehenden Ansprüche, wobei der C-terminale Teil aus dem Teil 585 bis 1578 von Humanplasmafibronectin oder einer Variante davon besteht.
- 25 5. Transformierter oder transfizierter Wirt mit einer Nukleotidsequenz, die so angeordnet ist, daß sie ein Fusionspolypeptid nach einem der vorhergehenden Ansprüche exprimieren kann.
- 30 6. Verfahren zur Herstellung eines Fusionspolypeptids durch Kultivieren eines Wirts nach Anspruch 5 und anschließendes Abtrennen des Fusionspolypeptids in einer geeigneten Form.
- 35 7. Fusionspolypeptid nach einem der Ansprüche 1 bis 4 zur therapeutischen Verwendung.

Patentansprüche für folgende Vertragsstaaten : ES, GR

- 35 1. Verfahren zur Herstellung eines Fusionspolypeptids durch
 - (i) Kultivieren eines transformierten oder transfektierten Wirts mit einer Nukleotidsequenz, die so angeordnet ist, daß sie ein Fusionspolypeptid exprimiert, und
 - (ii) anschließendes Abtrennen des Fusionspolypeptids in einer geeigneten Form,
 - 40 dadurch gekennzeichnet, daß das Fusionspolypeptid als mindestens einen Teil seines N-terminalen Teils einen N-terminalen Teil von HSA oder eine Variante davon und als mindestens einen Teil seines C-terminalen Teils ein weiteres Polypeptid umfaßt, mit der Ausnahme, daß wenn es sich bei dem N-terminalen Teil von HSA um den Teil 1-n mit n= 369 bis 419 oder eine Variante davon handelt, das Polypeptid aus
 - (a) dem Teil 585-1578 von Humanfibronectin oder einer Variante davon,
 - (b) dem Teil 1-368 von CD4 oder einer Variante davon,
 - (c) dem Platelet Derived Growth Factor oder einer Variante davon,
 - (d) dem Transforming Growth Factor β oder einer Variante davon,
 - (e) dem Teil 1-261 von reifem Humanplasmafibronectin oder einer Variante davon,
 - (f) dem Teil 278-578 von reifem Humanplasmafibronectin oder einer Variante davon,
 - (g) dem Teil 1-272 von reifem Human-von Willebrand's-Faktor oder einer Variante davon oder
 - (h) α -1-Antitrypsin oder einer Variante davon besteht.
 - 45 2. Verfahren nach Anspruch 1, wobei das Fusionspolypeptid zusätzlich mindestens eine N-terminale Aminosäure, die länger als der dem N-terminalen Teil von HSA entsprechende Teil ist, umfaßt.
 - 50 3. Verfahren nach Anspruch 1 oder 2, wobei sich in dem Fusionspolypeptid an der Verbindung der N-terminalen oder C-terminalen Teile eine spaltbare Region befindet.

4. Verfahren nach einem der vorhergehenden Ansprüche, wobei der C-terminale Teil aus dem Teil 585-1578 von Humanplasmafibronectin oder einer Variante davon besteht.

5 **Revendications**

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, DK, FR, IT, LU, NL, SE

1. Polypeptide fusionné comprenant en tant qu'au moins une partie de sa portion N-terminale, une portion N-terminale de HSA ou d'un variant de celle-ci et, en tant qu'au moins une partie de sa portion C-terminale, un autre polypeptide sauf que, lorsque cette portion N-terminale de HSA est la portion 1-n dans laquelle n est 369 à 419 ou un variant de celle-ci, ce polypeptide est (a) la portion 585 à 1578 de la fibronectine humaine ou un variant de celle-ci, (b) la portion 1 à 368 de CD4 ou un variant de celle-ci, (c) le facteur de croissance dérivé des plaquettes sanguines ou un variant de celui-ci, (d) le facteur de croissance β de transformation ou un variant de celui-ci, (e) la portion 1-261 de la fibronectine mature de plasma humain ou un variant de celle-ci, (f) la portion 278-578 de la fibronectine mature de plasma humain ou un variant de celle-ci, (g) la portion 1-272 du facteur humain mature de von Willebrand ou un variant de celle-ci, ou (h) l'alpha-1-antitrypsine ou un variant de celle-ci.
2. Polypeptide fusionné suivant la revendication 1, comprenant de plus au moins un acide aminé N-terminal se prolongeant au-delà de la portion correspondant à la portion N-terminale de HSA.
3. Polypeptide fusionné suivant les revendications 1 ou 2, dans lequel il y a une région susceptible d'être coupée à la jonction de ces portions N-terminale et C-terminale.
4. Polypeptide fusionné suivant l'une quelconque des revendications précédentes, dans lequel cette portion C-terminale est la portion 585 à 1578 de la fibronectine de plasma humain ou un variant de celle-ci.
5. Hôte transformé ou transfecté ayant une séquence de nucléotides arrangée de façon à exprimer un polypeptide fusionné suivant l'une quelconque des revendications précédentes.
6. Procédé pour préparer un polypeptide fusionné par culture d'un hôte suivant la revendication 5, suivie de la séparation du polypeptide fusionné sous une forme utile.
7. Polypeptide fusionné suivant l'une quelconque des revendications 1 à 4 utilisable en thérapie.

Revendications pour les Etats contractants suivants : ES, GR

1. Procédé pour préparer un polypeptide fusionné par (i) la culture d'un hôte transformé ou transfecté ayant une séquence de nucléotides arrangée de façon à exprimer un polypeptide fusionné, suivie de (ii) la séparation du polypeptide fusionné sous une forme utile, caractérisé en ce que le polypeptide fusionné comprend, en tant qu'au moins une partie de sa portion N-terminale, une portion N-terminale de HSA ou d'un variant de celle-ci et, en tant qu'au moins une partie de sa portion C-terminale, un autre polypeptide sauf que, lorsque cette portion N-terminale de HSA est la portion 1-n dans laquelle n est 369 à 419 ou un variant de celle-ci, ce polypeptide est alors (a) la portion 585 à 1578 de la fibronectine humaine ou un variant de celle-ci, (b) la portion 1 à 368 de CD4 ou un variant de celle-ci, (c) le facteur de croissance dérivé des plaquettes sanguines ou un variant de celui-ci, (d) le facteur de croissance β de transformation ou un variant de celui-ci, (e) la portion 1-261 de la fibronectine mature de plasma humain ou un variant de celle-ci, (f) la portion 278-578 de la fibronectine mature de plasma humain ou un variant de celle-ci, (g) la portion 1-272 du facteur humain mature de von Willebrand ou un variant de celle-ci, ou (h) l'alpha-1-antitrypsine ou un variant de celle-ci.
2. Procédé suivant la revendication 1, dans lequel le polypeptide fusionné comprend de plus au moins un acide aminé N-terminal se prolongeant au-delà de la portion correspondant à la portion N-terminale de HSA.
3. Procédé suivant les revendications 1 ou 2 dans lequel, dans le polypeptide fusionné, il y a une région susceptible d'être coupée à la jonction de ces portions N-terminale et C-terminale.

4. Procédé suivant l'une quelconque des revendications précédentes, dans lequel cette portion C-terminale est la portion 585 à 1578 de la fibronectine de plasma humain ou un variant de celle-ci.

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FIGURE 1

FIGURE 1 Cont.

390	400
Vai Glu Glu Pro Gin Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu	
410	
Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro Gln Val Ser Thr	420
430	
Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His	440
450	
Pro Glu Ala Lys Arg Met Pro Cys Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu	460
470	
Cys Val Leu His Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser	480
490	
Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr Tyr Val Pro Lys	500
510	
Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp Ile Cys Thr Leu Ser Glu Lys Glu	520
530	
Arg Gin Ile Lys Lys Gln Thr Ala Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr	540
550	
Lys Glu Gln Leu Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys	560
570	
Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val Ala Ala Ser Gln	580
590	
Ala Ala Leu Gly Leu	

FIGURE 2 DNA sequence coding for mature HSA

10	20	30	40	50	60	70	80
GATGCCACACAAGAGTGAGGTTGCTCATCGGTTAAAGATTTGGGAGAAGAAAATTCAAAAGCCTGGTGGTGAATTGCCCT							
D A S K S E V A H R F K D L G E E N F K A L V L I A F							
90	100	110	120	130	140	150	160
TGCTCAGTATCTTCAGCAGTGTCCATTGAGATCATGTAAGATGAACTGAACTGAAATTGCCAAAACATGTG							
A Q Y L Q Q C P F E D H V K L V N E V T E F A K T C							
170	180	190	200	210	220	230	240
TGGCTGATGAGTCAGCTGAAATTGTGACPAATCACTTCATACCCCTTTGGAGACAAATTATGCACACTGGCAACTCTT							
V A D E S A E N C D K S L H T L F G D K L C T V A T L							
250	260	270	280	290	300	310	320
CGTGAACCTATCGTGAATGGCTGACTGCTGTGCAAAACAGAACCTGAGAGAAATGAAATGCTTCTTGCACACAAAGA							
R E T Y G E M A D C C A K Q E P E R N E C F L Q H K D							
330	340	350	360	370	380	390	400
TGACAAACCCAAACCTCCCCGATTGGTGAGACCAAGAGTTGATGTGACTGCTTTCATGACAATGAAAGAGACAT							
D N P N L P R L V R P E V D V M C T A F H D N E E T							
410	420	430	440	450	460	470	480
TTTGAAAAAAATCTTATRTGAAATTGCCAGAAGACATCCTTACTTTATGCCCGGAACTCTCTTTCTTGCTAAAAAGG							
F L K K Y L Y E I A R R H P Y F Y A P E L L F F A K R							
490	500	510	520	530	540	550	560
TATAAACCTGCTTTACAGAATGTTGCCAAAGCTGCTGATAAAAGCTGCCCTGTTGCCAAAGCTCGATGAAACTTCGGGA							
Y X A A F T E C C Q A A D K A A C L L P K L D E L R D							
570	580	590	600	610	620	630	640
TGAAGGGAAAGGCTTCGTCTGCCAAACAGAGACTCAATGTGCCAGTCTCCAAAATTGGAGAAAGAGCTTCAAGCAT							
E G K A S S A K Q R L K C A S L Q K F G E R A F K A							
650	660	670	680	690	700	710	720
GGGCAGTGGCTCGCCTGAGCCAGAGATTCCAAAGCTGAGTTGCCAGAAGTTCCAAGTTAGTGCACAGATCTTACCAA							
W A V A R L S Q R F P K A E F A E V S K L V T D L T K							
730	740	750	760	770	780	790	800
GTCCACACGGAATGCTGCCATGGAGATCTGCTGATGACAGGGCGGACCTTGCCAACTATATCTGTGAAAAA							
V H T E C C H G D L L E C A D D R A D L A K Y I C E N							
810	820	830	840	850	860	870	880
TCAGGATTGATCTCCAGTAAACTGAAGGAATGCTGAAAAACCTCTGTTGGAAAAATCCCAC TGCAATTGCCGAAGTGG							
Q D S I S S K L K E C C E K P L L E K S H C I A E V							
890	900	910	920	930	940	950	960
AAAATGAGATGAGATGCCCTGCTGACTTGCCTCATAGCTGCTGATTTGTTGAAAGTAAGGATGTTGCCAAAACATATGCT							
E N D E M P A D L P S L A A D F V E S K D V C K N Y A							
970	980	990	1000	1010	1020	1030	1040
GAGGAAAGGATGTCCTGCCATGTTTGTATGAAATATGCAAGAAGGCATCCTGATTACTCTGTCGTGCTGCTGCT							
E A K D V F L G M F L Y E Y A R R H P D Y S V V L L L							

FIGURE 2 Cont.

1050 1060 1070 1080 1090 1100 1110 1120
 GAGACTTGC~~A~~AGACATATGAAACCAC~~T~~TAGAGAA~~G~~TGCTGTGCCGCTGCAGATCCTCATGAATGCTATGCC~~A~~AGTGT
 R L A K T Y E T T L E K C C A A A D P H E C Y A K V

 1130 1140 1150 1160 1170 1180 1190 1200
 TCGATGAATTAAACCTCTGTGGAAGAGCCTCAGAAATTAAATC~~A~~ACAA~~A~~ACTGTGAGCTTTGAGCAGCTTGGAGAG
 F D E F K P L V E E P Q N L I K Q N C E L F E Q L G E

 1210 1220 1230 1240 1250 1260 1270 1280
 T~~A~~CAAATTCCAGAATGCC~~T~~ATTAGTC~~G~~TTACACCAAGAA~~G~~TACCC~~A~~AGTGTCA~~A~~CTCCAA~~C~~TCTGTAGAGGTCTC
 Y K F Q N A L L V R Y T K K V P Q V S T P T L V E V S

 1290 1300 1310 1320 1330 1340 1350 1360
 AAGAAACCTAGGAAAAGTGGCAGCAAATGTTGTAACATCCTGAAGC~~A~~AAAGAATGCC~~C~~TGTGAGAAGACTATCTAT
 R N L G K V G S K C C K H P E A K R M P C A E D Y L

 1370 1380 1390 1400 1410 1420 1430 1440
 CCGTGGTC~~T~~GAACCGAGTATGTTGCTGAGAAAACGCCAGTAAGTGA~~C~~AGAGTCACAA~~A~~ATGCTGCACAGAGTC
 S V V L N Q L C V L H E K T P V S D R V T K C C T E S

 1450 1460 1470 1480 1490 1500 1510 1520
 TTGGTGAACAGGC~~G~~ACC~~A~~TGCTTTCAGCTCTGGAA~~G~~TGATGAAACATACGTTCCAAAGAGTTAATGCTGAACATT
 L V N R R P C F S A L E V D E T Y V P K E F N A E T F

 1530 1540 1550 1560 1570 1580 1590 1600
 CACCTTCATGCAGATATGCA~~C~~ACTTCTGAGAAGGAGAGACAA~~A~~ACTGCA~~A~~CTTGTGAGCTTGTG~~A~~
 T F H A D I C T L S E K E R Q I K K Q T A L V E L V

 1610 1620 1630 1640 1650 1660 1670 1680
 AACACAAAGCC~~A~~AGGC~~A~~ACAAAGAGCAACTGAAAGCTGTTATGGATGATTCCGAGCTTTGAGAGAA~~G~~TGCTGCAAG
 K H K P K A T K E Q L K A V M D D F A A F V E K C C K

 1690 1700 1710 1720 1730 1740 1750 1760
 CCTGACGATAAGGAGACCTGCTTGC~~C~~GAGGAGGGTAA~~A~~ACTGTGCTGCAAGTCAAGCTGCC~~T~~AGGCTTATAAC~~A~~
 A D D K E T C F A E E G K K L V A A S Q A A L G L

 1770 1780
 TCTACATTTAAAGC~~A~~TCTCAG

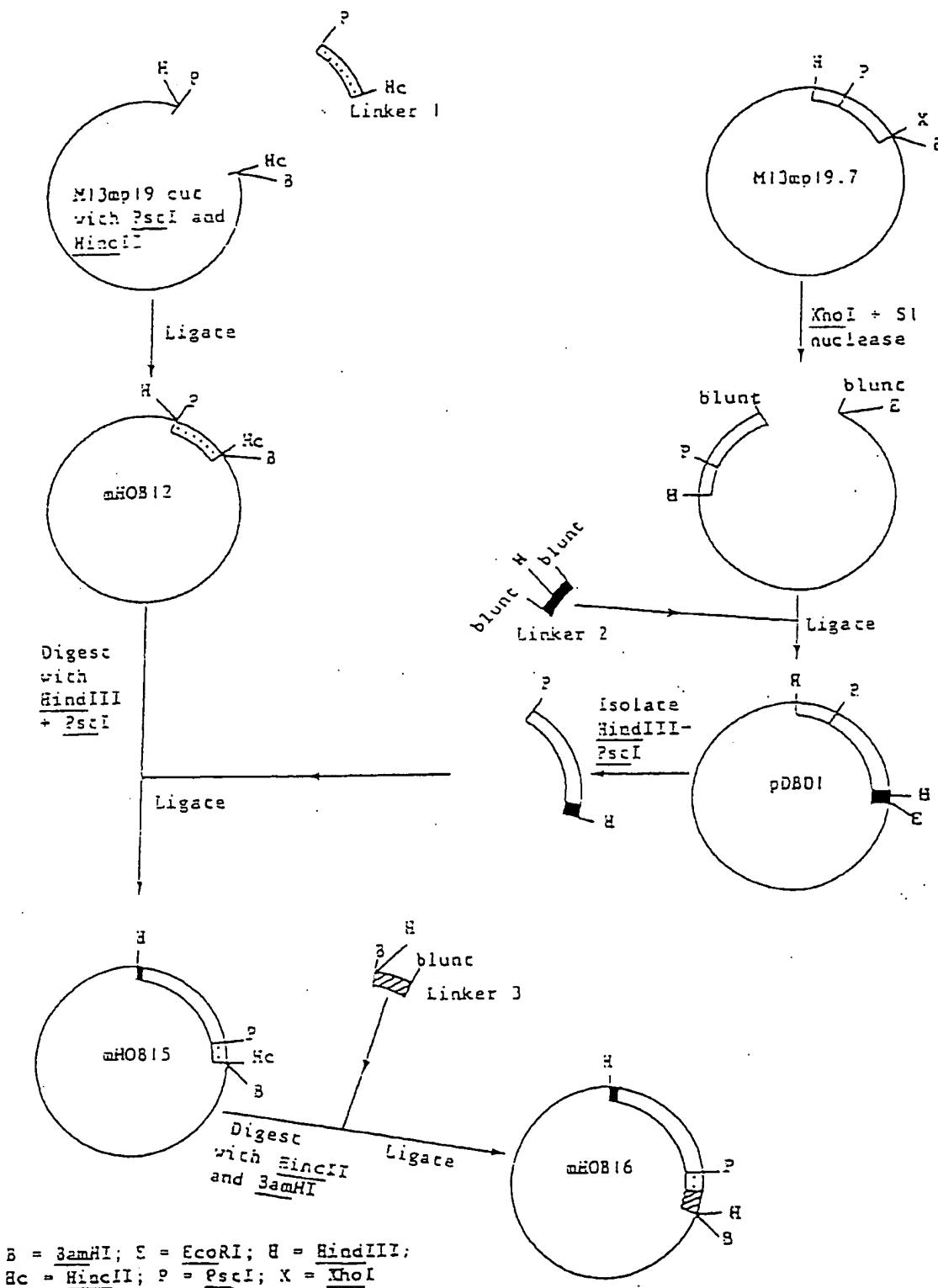
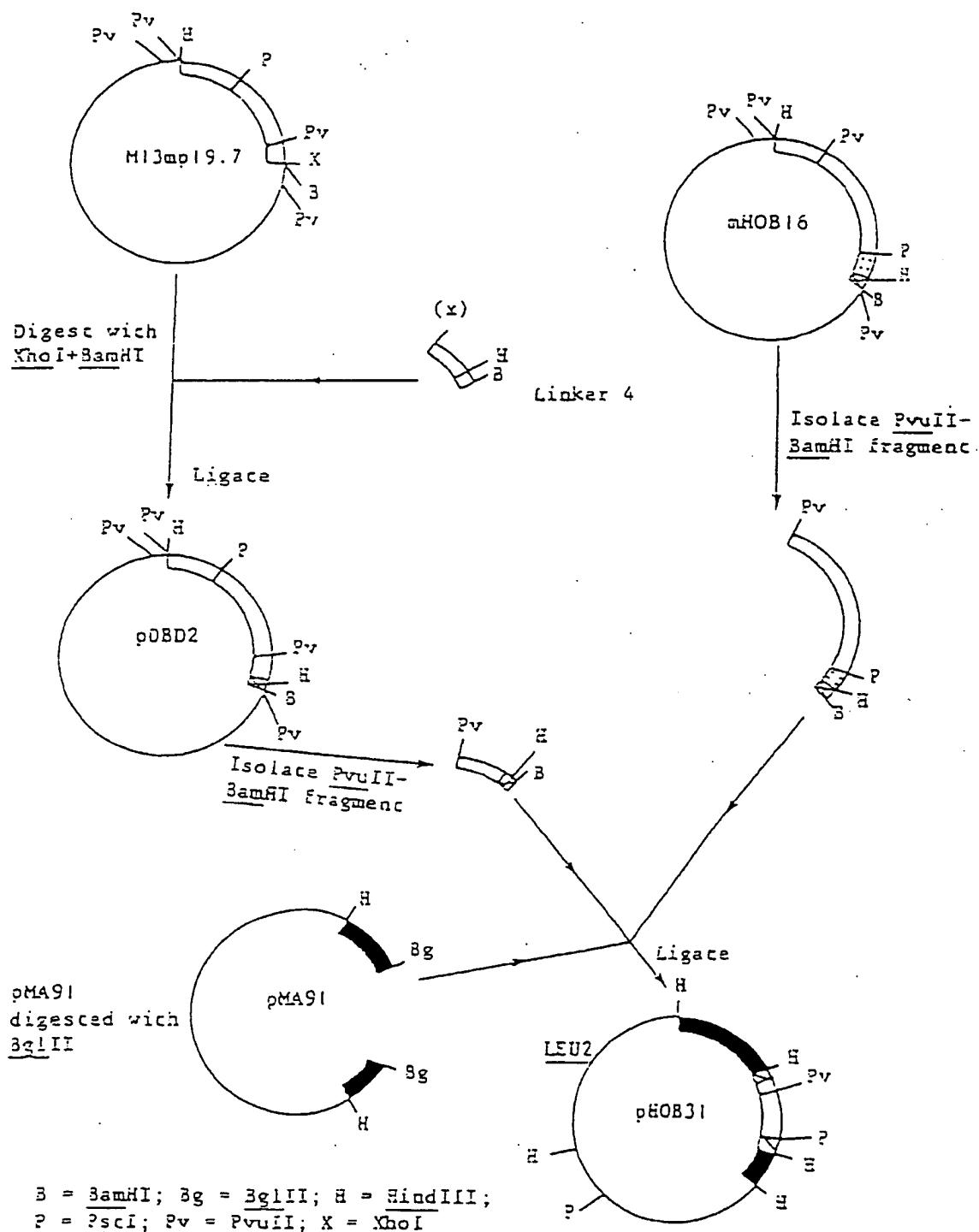
FIGURE 3 Construction of mHO816

FIGURE 4 Construction of pHOB31



Gln Ala Gin Met Val Gin Pro Gin Ser ¹⁰ Pro Val Ala Val Ser Gin Ser Lys Pro ²⁰ Gly
 Cys Tyr Asp Asn Gly Lys His Tyr Gin ³⁰ Ile Asn Gin Gin Trp Glu Arg Thr Tyr Leu Gly ⁴⁰
 Asn Val Leu Val Cys Thr Cys Tyr Gly ⁵⁰ Ser Arg Gly Phe Asn Cys Glu Ser Lys Pro ⁶⁰
 Glu Ala Glu Glu Thr Cys Phe Asp Lys ⁷⁰ Tyr Thr Gly Asn Thr Tyr Arg Val Gly Asp Thr ⁸⁰
 Tyr Glu Arg Pro Lys Asp Ser Met Ile Trp Asp Cys Thr Cys Ile Gly Ala Gly Arg Gly ⁹⁰
 Arg Ile Ser Cys Thr Ile Ala Asn Arg Cys His Glu Gly Gly ¹¹⁰ Gin Ser Tyr Lys Ile Gly ¹²⁰
 Asp Thr Trp Arg Arg Pro His Glu Thr Gly ¹³⁰ Tyr Met Leu Glu Cys Val Cys Leu Gly ¹⁴⁰
 Asn Gly Lys Gly Glu Trp Thr Cys Lys ¹⁵⁰ Pro Ile Ala Glu Lys Cys Phe Asp His Ala ¹⁶⁰
 Gly Thr Ser Tyr Val Val Gly Glu Thr Trp Glu Lys Pro Tyr Gin Gly Trp Met Met Val ¹⁷⁰
 Asp Cys Thr Cys Leu Gly Glu Gly Ser Gly ¹⁹⁰ Arg Ile Thr Cys Thr Ser Arg Asn Arg Cys ²⁰⁰
 Asn Asp Gin Asp Thr Arg Thr Ser Tyr Arg ²¹⁰ Ile Gly Asp Thr Trp Ser Lys Lys Asp Asn ²²⁰
 Arg Gly Asn Leu Leu Gin Cys Ile Cys ²³⁰ Thr Gly Asn Gly Arg Gly Glu Trp Lys Cys Glu ²⁴⁰
 Arg His Thr Ser Val Gin Thr Ser Ser Gly Ser Gly Pro Pro Val Arg ²⁵⁰ Val Arg ²⁶⁰
 Ala Val Tyr Gin Pro Gin Pro His Pro ²⁷⁰ Pro Pro Tyr Gly His Cys Val Thr Asp ²⁸⁰
 Ser Ely Val Val Tyr Ser Val Gly Met ²⁹⁰ Gin Trp Leu Lys Thr Gin Gly Asn Lys Gin ³⁰⁰
 Leu Cys Thr Cys Leu Gly Asn Gly Val Ser Cys Gin Glu Thr Ala Val Thr ³¹⁰ Gin ³²⁰
 Gly Gly Asn Ser Asn Gly Glu Pro Cys Val Leu Pro Phe Thr Tyr Asn Gly Arg Thr ³³⁰ Phe ³⁴⁰
 Tyr Ser Cys Thr Thr Glu Gly Arg Gly His Leu Trp Cys Ser Thr Thr Ser ³⁵⁰ Ser Asn ³⁶⁰
 Tyr Glu Glu Asp Gin Lys Tyr Ser Phe Cys Thr Asp His Thr Val Leu Val Gin Thr ³⁷⁰ Gin ³⁸⁰
 Gly Gly Asn Ser Asn Ely Ala Leu Cys His Phe Pro Phe Leu Tyr Asn Asn His Asn Tyr ³⁹⁰ Tyr ⁴⁰⁰
 Thr Asp Cys Thr Ser Gin Gly Arg Arg Asp Asn Met Lys Trp Cys Gly Thr Thr Gin Asn ⁴¹⁰ Asn ⁴²⁰

Fig. 5A

Tyr Asp Ala Asp Glu Lys Phe Gly Phe Gly Pro Met Ala Ala His Glu Glu Ile Cys ⁴³⁰
 Thr Asn Glu Gly Val Met Tyr Arg Ile ⁴⁵⁰Gly Asp Glu Trp Asp Lys Glu His Asp Met ⁴⁶⁰
 His Met Met Arg Cys Thr Cys Val Gly Asn Gly Arg Gly Glu Trp Thr Cys Tyr Ala ⁴⁸⁰
 Ser Glu Leu Arg Asp Glu Cys Ile Val Asp Asp Ile Thr Tyr Asn Val Asp Thr ⁵⁰⁰
 His Lys Arg His Glu Glu Gly His Met Leu Asn Cys Thr Cys Phe Gly Glu Arg Gly ⁵²⁰
 Arg Trp Lys Cys Asp Pro Val Asp Glu Cys ⁵³⁰Gly Asp Ser Glu Thr Gly Thr Phe Tyr
 Ile Gly Asp Ser Trp Glu Lys Tyr Val His ⁵⁵⁰Gly Val Arg Tyr Glu Cys Tyr Cys Tyr
 Arg Gly Ile Gly Glu Trp His Cys Glu ⁵⁷⁰Leu Glu Thr Tyr Pro Ser Ser Gly ⁵⁹⁰
 Val Val Phe Ile Thr Glu Thr Pro Ser ⁶⁰⁰Gly Pro Asn Ser His Pro Ile Glu Trp Asn
 Ala Pro Glu Pro Ser His Ile Ser Lys ⁶¹⁰Tyr Ile Leu Arg Trp Arg Pro Lys Asn Ser
 Gly Arg Trp Lys Glu Ala Thr Ile Pro ⁶³⁰Gly His Leu Asn Ser Tyr Thr Ile Lys Gly ⁶⁴⁰
 Lys Pro Gly Val Val Tyr Glu Gly Glu ⁶⁵⁰Ile Ser Ile Glu Glu Tyr Gly His Glu ⁶⁶⁰
 Val Thr Arg Phe Asp Phe Thr Thr Thr Ser ⁶⁷⁰Thr Ser Thr Pro Val Thr Ser Asn Thr Val
 Thr Gly Glu Thr Pro Phe Ser Pro ⁶⁹⁰Leu Val Ala Thr Ser Glu Ser Val Thr Glu ⁷⁰⁰
 Thr Ala Ser Ser Phe Val Val Ser Trp Val Ser Ala Ser Asp Thr Val Ser Gly ⁷¹⁰
 Val Glu Tyr Glu Leu Ser Glu Glu Gly ⁷³⁰Asp Glu Pro Glu Tyr Leu Asp Leu Pro Ser ⁷⁴⁰
 Ala Thr Ser Val Asn Ile Pro Asp Leu ⁷⁵⁰Leu Pro Ely Arg Lys Tyr Ile Val Asn Val ⁷⁶⁰
 Gin Ile Ser Glu Asp Gly Glu Gin Ser ⁷⁷⁰Leu Ile Leu Ser Thr Ser Glu Thr Thr Ala Pro ⁷⁸⁰
 Asp Ala Pro Pro Asp Pro Thr Val Asp Asp Asp Thr Ser Ile Val Val Arg Trp ⁸⁰⁰
 Ser Arg Pro Glu Ala Pro Ile Thr Gly ⁸¹⁰Tyr Arg Ile Val Tyr Ser Pro Ser Val Glu ⁸²⁰
 Ser Ser Thr Glu Leu Leu Asn Leu Pro Glu ⁸³⁰Thr Ala Asn Ser Val Thr Leu Ser Asp Leu Glu ⁸⁴⁰
 FNDL 1

Fig. 5B

Pro Gly Val Gin Tyr Asn Ile Thr Ile Val Glu Glu Asn Gln Glu Ser Thr 850
 Val Val Ile Gin Gin Glu Thr Thr Gly Thr Pro Arg Ser Asp Thr Val Pro Ser Pro 870
 Asp Val Lys Val Thr Asp Val Lys Val Thr Ile Met Trp Thr Pro Pro 890
 Ser Ala Val Thr Gly Tyr Arg Val Asp Val Ile Pro Val Asn Leu Pro Gly Glu His 910
 Gln Arg Leu Pro Ile Ser Arg Asn Thr Phe Ala Glu Val Thr Gly Leu Ser Pro Gly 930
 Val Thr Tyr Phe Lys Val Phe Ala Val Ser His Gly Arg Glu Ser Lys Pro Leu Thr 950
 Ala Gln Gin Thr Lys Leu Asp Ala Pro Thr Asn Leu Gln Phe Val Asn Glu Thr Asp 970
 Ser Val Leu Val Arg Trp Thr Pro Pro 990 Ala Gin Ile Thr Gly Tyr Arg Leu Thr 1000
 Val Gly Leu Thr Arg Arg Gly Gin Pro Arg Gin Tyr Asn Val Gly Pro Ser Val Ser Lys 1010
 Tyr Pro Leu Arg Asn Leu Gln Pro Ala Ser Gln Tyr Thr Val Ser Leu Val Ala Ile Lys 1030
 Asn Gln Glu Ser Pro Lys Ala Thr Gly Val Phe Thr Thr Leu Gln Pro Gly Ser Ser 1050
 Ile Pro Pro Tyr Asn Thr Glu Val Thr Glu Thr Thr Ile Val Ile Thr Trp Thr Pro Ala 1070
 Pro Arg Pro Ser Gln Gly Gly Ala Pro Arg Glu Val 1090
 Arg Ile Gly Phe Lys Leu Gly Val Arg Asp Ser Gln Glu Ala Pro Arg Glu Val 1110
 Thr Ser Asp Ser Ile Val Val Ser Gly Leu Thr Pro Gly Val Glu Tyr Val Tyr 1130
 Thr Ile Gin Val Leu Arg Asp Gly Gln Glu Arg Asp Ala Pro Ile Val Asn Lys Val Val 1150
 Thr Pro Leu Ser Pro Pro Thr Asn Leu His Leu Glu Ala Asn Pro Asp Thr Gly Val Leu 1170
 Thr Val Ser Trp Glu Arg Ser Thr Thr Pro Asp Ile Thr Gly Tyr Arg Ile Thr Thr 1180
 Thr Pro Thr Asn Gly Gin Gln Asn Ser Leu Glu Glu Val His Ala Asp Gln Ser Ser 1190
 Cys Thr Phe Asp Asn Leu Ser Pro Gly Leu Glu Tyr Asn Val Ser Val Tyr Thr Val Lys 1210 1220
 Asp Asp Lys Glu Ser Val Pro Ile Ser Asp Thr Ile Ile Pro Ala Val Pro Pro Pro Thr 1230 1240
 Asp Asp Leu Arg Phe Thr Asn Ile Gly Pro Asp Thr Met Arg Val Thr Trp Ala Pro Pro Pro 1250 1260

Fig. 5C

Ser Ile Asp Leu Thr Asn Phe Leu Val Arg Tyr Ser Pro Val Lys Asn Glu Glu Asp Val 1270
 Ala Glu Leu Ser Ile Ser Pro Ser Asp Asn Ala Val Val Leu Thr Asn Leu Leu Pro Gly 1290
 Thr Glu Tyr Val Val Ser Val Ser Val Tyr Glu Glu Ser Thr Pro Leu Arg 1310
 Gly Arg Gln Lys Thr Gly Leu Asp Ser Pro Thr Gly Ile Asp Phe Ser Asp Ile Thr Ala 1330
 Asn Ser Phe Thr Val His Trp Ile Ala pro Arg Ala Thr Ile Thr Gly Tyr Arg Ile Arg 1350
 HIS HIS Pro Glu His Phe Ser Gly Arg Pro Glu Asp Arg Val Pro His Ser Arg Asn 1370
 Ser Ile Thr Leu Thr Asn Lys Thr Pro Gly Thr Glu Tyr Val Val Ser Ile Val Ala Leu 1390
 Asn Gly Arg Glu Glu Ser Pro Leu Leu Ile Gly Glu Ser Thr Val Ser Asp Val Pro 1410
 Arg Asp Leu Glu Val Ala Ala Thr Pro Thr Ser Leu Leu Ile Ser Trp Asp Ala Pro 1430
 Ala Val Thr Val Arg Tyr Tyr Ile Thr Tyr Gly Glu Thr Gly Asn Ser Pro Val 1450
 Gln Glu Phe Thr Val Pro Gly Ser Lys Ser Thr Ala Thr Ile Ser Gly Leu Lys Pro Gly 1470
 Val Asp Tyr Thr Ile Thr Val Tyr Ala Val Thr Gly Arg Gly Asp Ser Pro Ala Ser Ser 1490
 Lys Pro Ile Ser Ile Asn Tyr Arg Thr Glu Ile Asp Lys Pro Ser Gln Met Gln Val Thr 1510
 Asp Val Gln Asp Asn Ser Ile Ser Val Lys Trp Leu Pro Ser Ser Pro Val Thr Gly 1530
 Tyr Arg Val Thr Thr Pro Lys Asn Gln pro Gly Pro Thr Lys Thr Ala Val Thr 1550
 Pro Asp Gln Thr Glu Met Thr Ile Glu Gln Leu Gln Pro Thr Val Glu Tyr Val Val Ser 1570
 Val Tyr Ala Gln Asn Pro Ser Gly Glu Ser Gln Pro Leu Val Gln Thr Ala Val Thr 1590
 Ile Pro Ala Pro Thr Asp Leu Lys Phe Thr Gln Val Thr Pro Thr Ser Leu Ser Ala Gln 1610
 Trp Thr Pro Pro Asn Val Gln Leu Thr Tyr Arg Val Arg Val Thr Pro Lys Glu Lys 1630
 Thr Gln Pro Met Lys Glu Ile Asn Leu Ala Pro Asp Ser Ser Val Val Ser Gly 1650
 Leu Met Val Ala Thr Lys Tyr Glu Val Ser Val Tyr Ala Leu Lys Asp Thr Leu Thr Ser 1670
 FNDEL 1

Fig. 5D

Arg Pro Ala Gin Gly Val Val Thr Thr Thr Leu Glu Asn Val Ser Pro Pro Arg Arg Arg Ala Arg
 Val Thr Asp Ala Thr Glu Thr Thr Ile Ser Trp Arg Thr Lys Thr Glu Thr Ile Arg Ala Arg
 Thr Gly Phe Gin Val Asp Ala Val Pro Ala Asn Gly Gin Thr Pro Ile Gin Arg Thr Ile Arg
 Lys Pro Asp Val Arg Ser Tyr Thr Ile Thr Gly Leu Gin Pro Gly Thr Asp Tyr Lys Ile Arg
 Tyr Leu Tyr Thr Leu Asn Asp Ala Arg Ser Ser Pro Val Val Ile Asp Ala Ser Thr Ile Arg
 Ala Ile Asp Ala Pro Ser Asn Lau Arg Phe Lau Ala Thr Thr Pro Asn Ser Leu Leu Val Arg
 Ser Trp Gin Pro Pro Arg Ala Arg Ile Thr Gly Tyr Ile Ile Lys Tyr Glu Lys Pro Gly Arg
 Ser Pro Pro Arg Glu Val Val Pro Arg Pro Gly Val Thr Glu Ala Thr Ile Thr Ile Arg
 Gly Leu Glu Pro Gly Thr Glu Tyr Thr Ile Tyr Val Ile Ala Leu Lys Asn Asn Gin Lys Arg
 Ser Glu Pro Leu Ile Gly Arg Lys Lys Thr Asp Glu Leu Pro Gin Leu Val Thr Leu Pro Arg
 His Pro Asn Leu His Gly Pro Glu Ile Leu Asp Val Pro Ser Thr Val Gin Lys Thr Pro Arg
 Phe Val Thr His Pro Gly Tyr Asp Thr Gly Asn Gly Ile Gin Leu Pro Gly Thr Ser Gly Arg
 Gin Gin Pro Ser Val Gly Gin Met Ile Phe Glu Glu His Gly Phe Arg Arg Thr Thr Ser Gly Arg
 Pro Pro Thr Thr Ala Thr Pro Ile Arg His Arg Pro Tyr Pro Pro Asn Val Ala Arg
 Leu Ser Gin Thr Thr Ile Ser Trp Ala Pro Phe Gin Asn Thr Ser Glu Tyr Ile Ile Ser Thr
 Cys His Pro Val Gly Thr Asp Glu Glu Pro Leu Gin Phe Arg Val Pro Gly Thr Ser Thr
 Ser Ala Thr Leu Thr Gly Leu Thr Arg Gly Ala Thr Tyr Asn Ile Ile Val Glu Ala Leu
 Lys Asp Gin Gin Arg His Lys Val Arg Elu Glu Val Val Thr Val Gly Asn Ser Val Asn
 Glu Gly Leu Asn Gin Pro Thr Asp Asp Ser Cys Phe Asp Pro Tyr Thr Val Ser His Tyr
 Ala Val Gly Asp Glu Trp Glu Arg Met Ser Glu Ser Gly Phe Lys Leu Leu Cys Gin Cys
 Leu Ser Phe Gly Ser Gly His Phe Arg Cys Asp Ser Ser Arg Trp Cys His Asp Asn Gly

Fig. 5E

Val Asn Tyr Lys Ile Gly Glu Lys Trp Asp Arg Gln Gly Glu Asn Gln Met Met Ser
 2110
 Cys Thr Cys Leu Gly Asn Gly Lys Gly Glu Phe Lys Cys Asp Pro His Glu Ala Thr Cys
 2130
 Tyr Asp Asp Gly Lys Thr Tyr His Val Gly Glu Gln Trp Gln Lys Glu Tyr Leu Gly Ala
 2150
 Ile Cys Ser Cys Thr Cys Phe Gly Gly Gln Arg Gly Trp Arg Cys Asp Asn Cys Arg Arg
 2170
 Pro Gly Glu Pro Ser Pro Glu Gly Thr Gln Ser Tyr Asn Gln Tyr Ser Gln
 2190
 Arg Tyr His Gln Arg Thr Asn Thr Asn Val Asn Cys Pro Ile Glu Cys Phe Met Pro Leu
 2210
 Asp Val Gln Ala Asp Arg Glu Asp Ser Arg Glu
 2230

Fig. 5F

1

GAAGAGCCTCAGAATTAAATCACTGAGACTCCGAGTCAGCCCCAACTCCCAACCCATCCAGTGG
CTTCTCGGAGTCTTAAATTAGTGACTCTGAGGCTCAGTCGGGTTGAGGGTGGGTAGGTCAACC

e e p q n l i t e | t p s q p n s h p i q w

8

3

AATGCACCACAGCCATCTCACATTCCAAGTACATTCTCAGGTGGAGACCTAAAAATTCTGTA
TTACGTGGTGTGGTAGAGTGTAAAGGTTCATGTAAGAGTCACCTCTGGATTAAAGACAT

n a p q p | s h i s k y i l r w r p k n s v

7

4

GGCCGTTGGAAGGAAGCTACCATACCGAGGCCACTTAAACTCCTACACCATCAAAGGCCTG
CCGGCAACCTTCCTCGATGGTATGGTCCGGTGAATTGAGGATGTGGTAGTTCCGGACTTAA

g | r w k e a t i p g h l n s | y t i k g l

6

5

Figure 6 Linker 5 showing the eight constituent oligonucleotides

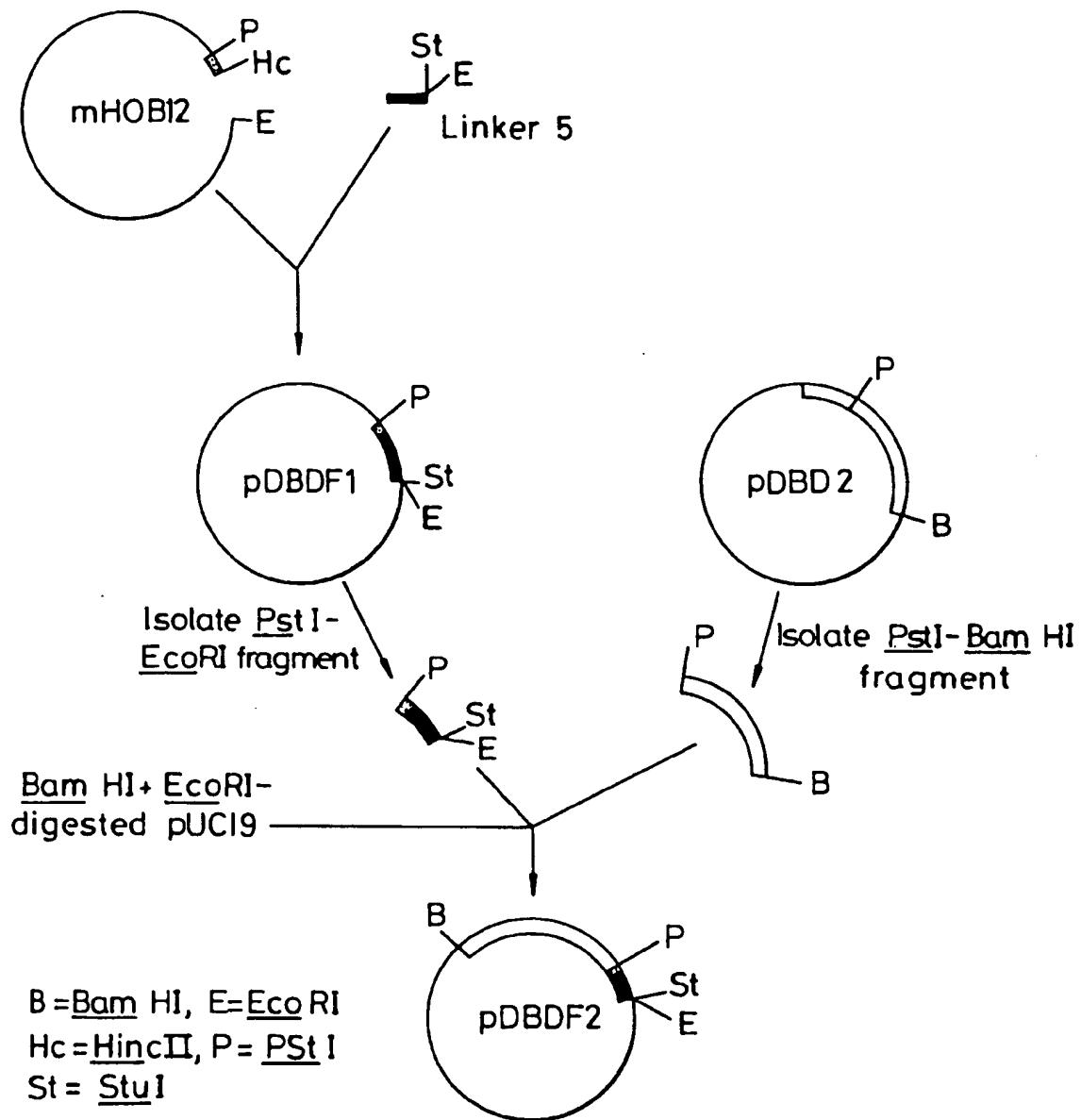


Fig. 7 Construction of pDBDF2

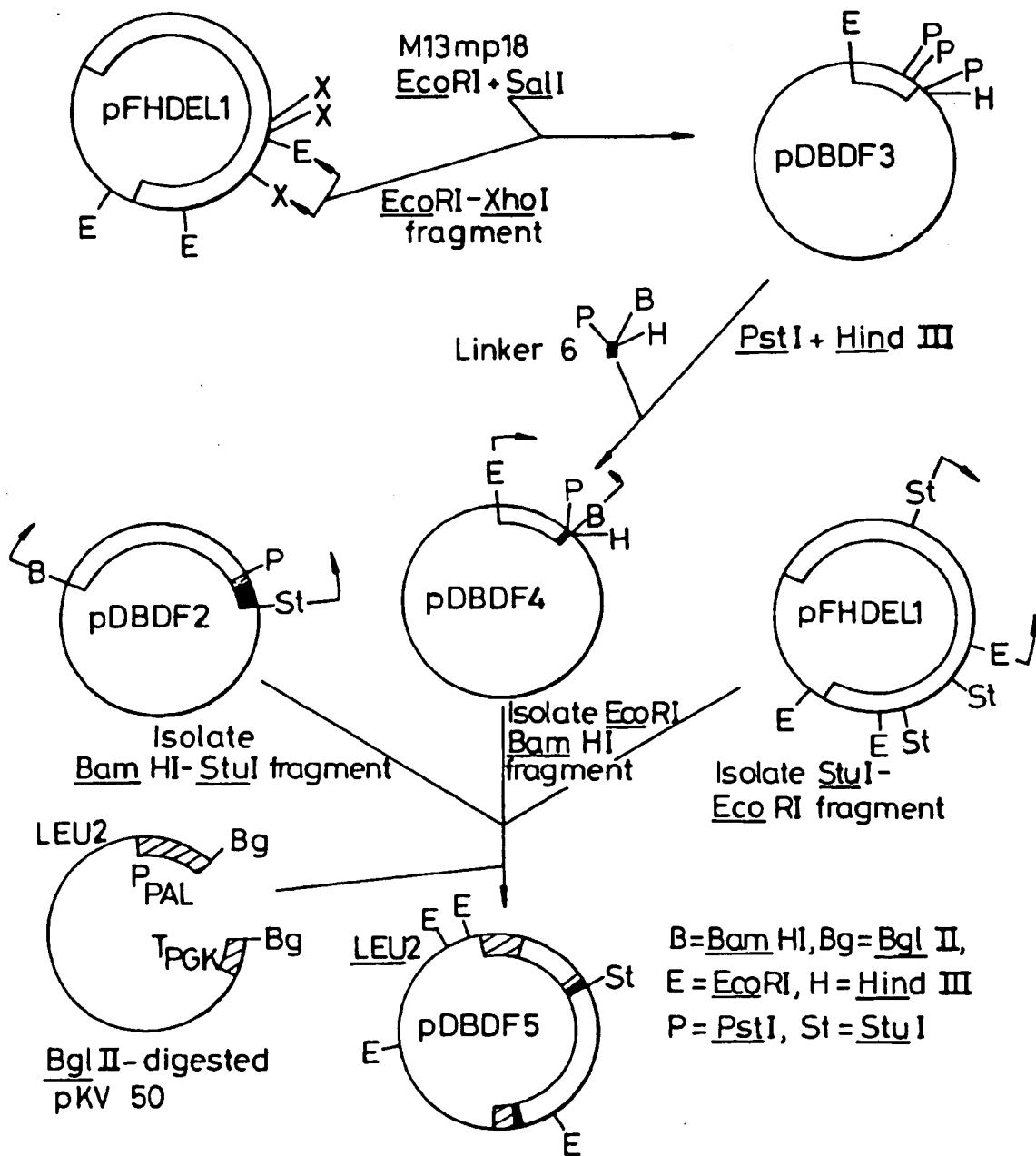


Fig. 8 Construction of pDBDF5

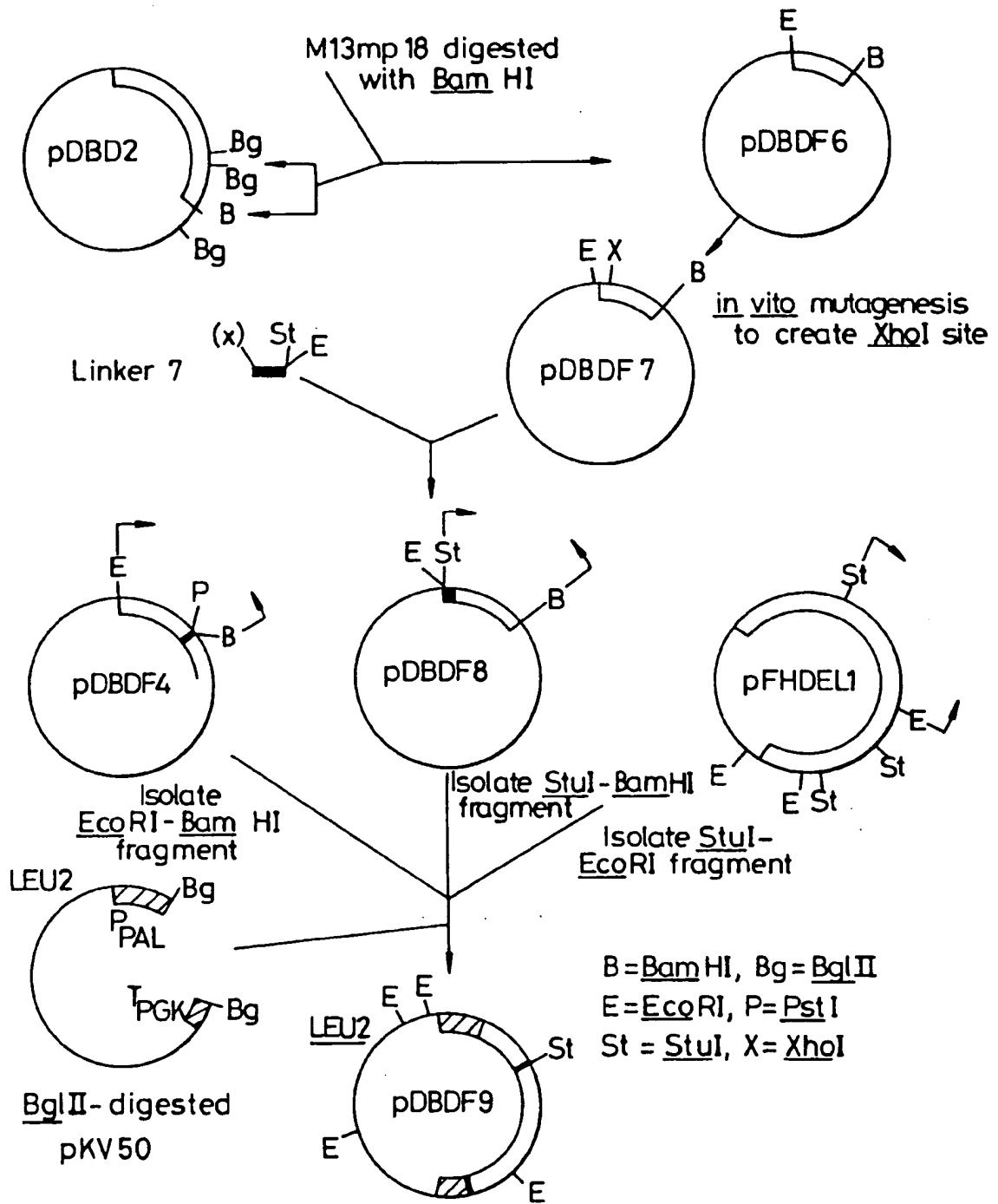


Fig. 9 Construction of pDBDF9

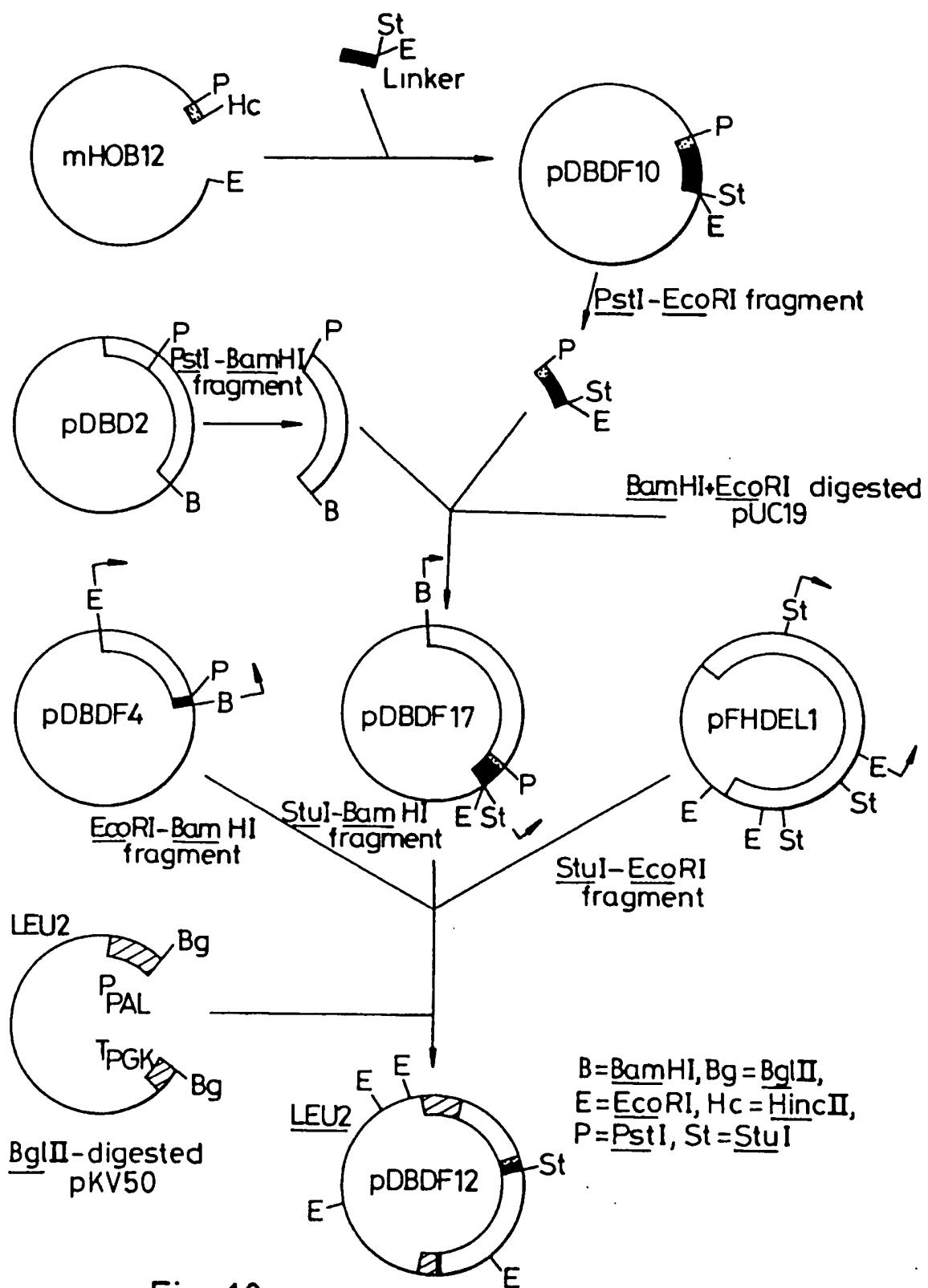


Fig. 10 Construction of pDBDF12

Figure 11

Name: pFHDEL1
 Vector: pUC18 Amp^r 2860bp
 Insert: hFNcDNA ~ 7630bp

